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(54) Title: METHOD FOR REMOVING DNA FROM PROTEIN PREPARATIONS

(57) Abstract

A method for removing DNA from protein preparations is disclosed. A purified or partially purified protein preparation is incubated with a secondary or quaternary amine anion exchanger for an extended period of time, at high pH values and low salt concentrations, to yield a product suitable for human or animal use.

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METHOD FOR REMOVING DNA FROM PROTEIN PREPARATIONS

Technical Field

The present invention relates generally to a method for removing DNA from protein preparations. More particularly, the present invention relates to the use of anion exchange resins to reduce the amount of DNA in injectables to levels considered safe.

Background of the Invention

Injectable protein preparations such as monoclonal antibodies, pharmaceuticals, vaccines and other compositions, should be rid of substantial amounts of DNA prior to their use. DNA contamination in such preparations can deleteriously affect the subject to whom they are administered. For instance, unwanted DNA segments might become incorporated into the recipient's genome. The FDA recommends that DNA levels in these preparations be 10 picograms per dose or less.

Previous methods of protein purification generally effect the gross removal of DNA early in the purification scheme. Many of these processes do not achieve the low DNA levels required for injectable grade proteins. Furthermore, column chromatography and other commonly used protein purification techniques often fail to remove maximal amounts of DNA due to its slow kinetics of adsorption.

The electric charge present on proteins in solution is known to be a function of several parameters including pH and ionic strength. The pH at which a protein

has no net electric charge is its isoelectric pH. The isoelectric pH varies from protein to protein.

The phosphate groups of DNA are fully ionized at any pH above 4. DNA is thus strongly acidic. Because of this property, DNA will bind positively charged groups. Additionally, when pH values are increased, more efficient binding can be effected. Proteins are also negatively charged above their isoelectric point and thus bind positively charged groups at high pH values. However, salt concentrations can be adjusted so that only minimal amounts of protein will bind these groups at elevated pH values. Thus, a delicate balance must be struck in order to achieve maximum DNA removal from a protein solution without the concomitant loss of significant amounts of protein.

Summary of the Invention

The present invention is based on the discovery that the prolonged contact of protein preparations with anion exchange resins, under suitable conditions, can remove significant amounts of DNA while rendering acceptable protein yields. Thus, a commercially acceptable protein preparation can be produced for use in humans and animals.

In one embodiment, the present invention is directed to a method for removing DNA from a protein preparation wherein the protein preparation is incubated with an anion exchange resin for a period of time and under conditions sufficient to result in a final protein product containing less than 5 picograms of DNA per milligram of product.

In another embodiment of the instant invention, a method for removing DNA from a protein preparation is provided, the method comprising incubating the preparation with an anion exchange resin under conditions such that DNA from the protein preparation becomes bound to the

resin while substantial amounts of protein from the protein preparation do not bind the resin.

In several preferred embodiments of the subject invention, the anion exchange resin comprises a secondary or quaternary anion exchange resin and incubation is carried out at a pH greater than 7, the pH preferably being other than the isoelectric pH of the desired protein. Salt concentrations are kept below about 500mM and incubation proceeds for more than about 2 hours.

Further embodiments of the present invention will occur to those of ordinary skill in the art.

Detailed Description

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

"Protein preparation" refers to any composition, solution or formulation, regardless of purity, that contains one or more peptides, polypeptides or proteins. Such a preparation may contain significant amounts of other non-protein substances. Exemplary protein preparations include but are not limited to polyclonal and monoclonal antibody-containing compositions, antigens, drugs, diagnostics, vaccines, injectables, pharmaceutical compounds and other therapeutic agents.

The "isoelectric pH" of a protein is the pH at which the protein exhibits no net electric charge and does not move in an electric field. The isoelectric pH of many proteins is known or can be readily determined by methods well known in the art.

By "substantial amounts of protein" remaining unbound to an exchange resin as contemplated by the instant invention, is meant that at least 50%, preferably 75% and more preferably 80% or more of the desired protein does not become bound to the exchanger.

A "substantially DNA-free" product is one that contains picogram quantities or less of DNA per mg of product. Preferably, a substantially DNA-free product will contain less than about 50 picograms per mg of product, more preferably, less than about 20 picograms per mg of product, and most preferably, less than about 10 picograms per mg of product.

An "anion exchange resin" comprises a matrix, such as agarose, cellulose, dextran, silica, and other synthetic polymers, to which secondary, tertiary or quaternary amine groups can be covalently bound. These exchangers are commercially available from several suppliers. Representative anion exchange resins include but are not limited to diethylaminoethyl (DEAE)-substituted materials such as DEAE-substituted cellulose, agarose, or dextran; or quaternary ammonium substituted resins such as Q-Sepharose and QAE-Sephadex, all of which are available from Pharmacia.

The substituent groups of anion exchangers are positively charged in solution, the degree of ionization being dependent on the pH. Hence, negatively charged molecules will adsorb to these exchange resins. DEAE-substituted materials are only partially ionized in normal operating buffers around pH 6-9, whereas quaternary ammonium groups remain ionized even at pH 12. Scopes, R.K., Protein Purification Principles and Practice, 2d. edition (Springer-Verlag 1987). This is in part due to the higher positive charge density present on the quaternary amine groups as opposed to secondary amines. Thus, quaternary amine anion exchangers are potentially more efficient at binding the negatively charged phosphate groups of DNA.

B. General Method

The instant invention yields a substantially DNA-free protein preparation, suitable for injectable use. DNA levels can be reduced to picogram amounts or less

using the following procedure. A protein-containing preparation at any stage of purity is incubated with an anion exchange resin. Suitable resins include secondary and quaternary amine substituted resins with the quaternary amine resins being preferred. Particularly useful is Q Sepharose Fast Flow (Pharmacia). This resin is a strong anion exchanger with a cross-linked agarose matrix and is stable over a wide range of pH values.

The amount of resin used per mg of protein preparation need only be an amount that provides sufficient binding sites for the DNA present in the preparation. Amounts on the order of 1 ml resin per mg of product, more usually 1 ml resin per 10-20 mg product, will find use with the present invention. Other suitable ratios are readily determinable by one of ordinary skill in the art.

A protein preparation containing a desired protein is incubated with the resin using a batch process. In this way, the preparation is allowed to remain in contact with the resin for an extended period of time, thus maximizing DNA removal by adsorption of the DNA to the anion exchanger. Incubation should proceed for at least 2 hours, more preferably 4-24 hours or more. When secondary amine anion exchange resins are used, longer incubation periods may be necessary in order to effect maximum DNA removal. Such extended incubation favors greater binding due to the slow kinetics of adsorption demonstrated by DNA.

In order to maximize DNA removal and minimize protein loss, incubation should be carried out at high pH values and low salt concentrations, such that the desired protein will remain free from the resin and not become denatured. The capacity of the anion exchangers used in the present invention to bind DNA increases with increasing pH. Similarly, proteins will have a tendency to bind to the resin at high pH values. Salt concentrations can be adjusted so that only minimal amounts of protein will

bind the resin. However, high salt concentrations can cause proteins to precipitate, resulting in low product yields. Thus, a balance must be struck between the pH levels and salt concentrations used in the instant method.

Preferably, incubation will be carried out at the highest possible pH and the lowest possible salt concentration that will effect maximum DNA binding and minimum protein binding. These parameters will vary according to the specific protein in question. Generally, pH values greater than 6, more preferably between 7 and 9, and most preferably 8.0 to 8.5, achieve the desired results. Suitable buffers include standard running buffers such as Tris or phosphate, with a salt concentration of less than 500mM. It is thus apparent that the combination of prolonged exposure, pH values and salt concentrations all contribute to the effectiveness of the instant invention.

After incubation, DNA levels in the product can be determined using any standard assay. Particularly useful is a DNA probe hybridization assay. Such an assay is commercially available as a kit from Oncor (Maryland) and is sensitive to 2-5 picograms of DNA.

Generally, probes are made from DNA purified from individual cell lines from which the product is derived. However, DNA from any source is suitable so long as it is cross-reactive with the DNA from the protein preparation. DNA can be isolated from such cells by phenol extraction or other procedures well known in the art. See, e.g., Maniatis, Fritsch & Sambrook, MOLECULAR CLONING: A LABORATORY MANUAL (1982); the series, METHODS IN ENZYMOLOGY (S. Colowick and N. Kaplan eds., Academic Press, Inc.) The isolated DNA is then biotin-labeled and hybridization and detection performed according to the manufacturer's directions. The isolated DNA is also used to prepare standards. The sample can be compared to these standards in order to determine the amount of DNA present. This process yields protein preparations with DNA concen-

trations at or lower to the sensitivity limits of the above described assay. Thus, the resulting product contains less than 2-5 picograms of DNA. Such low DNA levels are commercially desirable and render the preparation useful as an injectable grade protein.

C. Examples

C.1. A purified murine immunoglobulin, derived from Invitron cell line 5F, was assayed to determine the amount of DNA present in the preparation prior to treatment with the anion exchanger via the following method. DNA was purified from this cell line using standard procedures. The DNA was biotin-labeled using the Oncor (Maryland) non-isotopic system for DNA probe labeling according to the manufacturer's directions, with the exception that the probe purification step was left out. Biotin labeling, using this system, is accomplished by incorporating biotin modified nucleotides into the DNA molecules using a standard nick translation technique.

Samples to be assayed were diluted 1:1 in a 4.5M urea solution, pH 13.0. Subsequent 1:10 dilutions were made in the same buffer. The diluted samples were transferred to a hybridization membrane and hybridization and detection performed using the Oncor non-isotopic system according to the manufacturer's directions. Briefly, this system utilizes streptavidin to bind the biotin-labeled DNA. Unbound streptavidin is removed and biotin-labeled alkaline phosphatase added to bind remaining available sites. Hybridized DNA is detected by incubation with 5-Bromo-4-Chloro-3-Indolyl Phosphate and Nitro Blue Tetrazolium which react in the presence of DNA to produce a blue-colored band. The intensity of the band varies with the amount of DNA present.

DNA standards were made by diluting known quantities of isolated DNA in the urea buffer described above. The standards were assayed as described above. The pre-

treated purified immunoglobulin sample was compared with the standards and found to contain 200-500 picograms of DNA per milligram of purified protein.

The purified murine immunoglobulin was then batched with Q Sepharose Fast Flow (Pharmacia), a quaternary ammonium-substituted cross-linked agarose, at a ratio of 20mg of immunoglobulin per ml of resin. A 20mM Tris buffer was used containing 90mM NaCl, pH 8.0. Incubation proceeded for 2 hours, 4 hours, or overnight. The DNA concentration in the treated protein preparations was determined as described above. DNA was reduced at least 100-fold in all three cases, to 2-5 picograms DNA per mg of product or lower. DNA amounts were at the level of sensitivity of the hybridization assay. Protein loss was between 5-10%. Thus, this treatment yielded a protein preparation suitable for human or animal use.

To test whether this method was effective in removing DNA during earlier stages in the purification process, partially purified murine immunoglobulin was treated as above. After the DNA removal step, the purification process was resumed. The final product was tested for DNA and had no detectable DNA using the hybridization method described. Thus, use of this invention during purification proved as efficacious as treatment of the final purified protein product.

To confirm the amount of DNA removed from the protein preparation using the foregoing method, bound DNA can be eluted from the anion exchange resin using a 1M NaCl wash followed by a .1M NaOH wash. The DNA can be assayed and quantified as described above. Similarly protein concentrations of the pre- and post-treated preparation can be determined via UV absorption, the Lowry or Biuret methods, or any other well known assay, and the values compared to determine the percent of protein loss.

C.2. A purified murine immunoglobulin, Subclass IgG_{2b}, derived from Invitron cell line 8B, was assayed to determine the amount of DNA present in the preparation prior to treatment with the anion exchanger as in example C.1. The sample contained 50-200 picograms of DNA per milligram of purified protein. This product was batched with Q Sepharose Fast Flow at a ratio of 15mg immunoglobulin per ml of resin as described above except that 20mM phosphate, 200mM NaCl, pH 8.5 was used as buffer. In each case, the process reduced DNA greater than 10-fold, to undetectable levels, when assayed as described in example C.1.

The experiment was repeated using only partially purified murine immunoglobulin as described in example C.1. Again, the method proved to be as effective when used during the purification process as it was when used after protein purification.

While the present invention has been illustrated above by certain specific embodiments, it is not intended that these specific examples limit the scope of the invention as described in the appended claims.

What is claimed is:

1. A method for removing DNA from a protein preparation comprising incubating said protein preparation with an anion exchange resin, said incubating being done for a period of time and under conditions sufficient to result in a final protein product containing less than about 5 picograms of DNA per milligram of product.

2. The method of claim 1 wherein said anion exchange resin is selected from the group consisting of secondary and quaternary anion exchange resins.

3. The method of claim 2 wherein said incubating is done at a pH greater than about 6.

4. The method of claim 2 wherein said incubating is done at a pH between approximately 7 and 9.

5. The method of claim 4 wherein said incubating is done at a pH other than the isoelectric pH of a protein of interest within said protein preparation.

6. The method of claim 2 wherein said incubating is done at a salt concentration of less than about 500mM.

7. The method of claim 2 wherein said incubating is done for more than about 2 hours.

8. The method of claim 2 wherein said incubating is done for more than about 4 hours.

9. A method for removing DNA from a protein preparation comprising incubating said protein preparation with a quaternary amine anion exchange resin for more than about 2 hours, said incubating being done at a pH greater

than about 6 and a salt concentration of less than about 500mM, said incubating resulting in a final protein product containing less than 5 picograms of DNA per milligram of product.

10. A method for removing DNA from a protein preparation comprising incubating said protein preparation with an anion exchange resin, said incubating being done under conditions such that DNA from said protein preparation binds to said resin and substantial amounts of protein from said protein preparation do not bind to said resin, said incubation resulting in a final protein product substantially free of DNA.

11. The method of claim 10 wherein said incubating results in a final protein product containing less than 5 picograms of DNA per milligram of product.

12. The method of claim 10 wherein said anion exchange resin is selected from the group consisting of secondary and quaternary anion exchange resins.

13. The method of claim 12 wherein said incubating is done at a pH greater than about 6.

14. The method of claim 12 wherein said incubating is done at a pH between approximately 7 and 9.

15. The method of claim 14 wherein said incubating is done at a pH other than the isoelectric pH of a protein of interest within said protein preparation.

16. The method of claim 12 wherein said incubating is done at a salt concentration of less than 500mM.

17. The method of claim 12 wherein said incubating is done for more than about 2 hours.

18. The method of claim 11 wherein said incubating is done for more than about 4 hours.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00409

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 3/20 3/22 C12N 15/10

U.S. Cl. 530/416, 412, 417 435/91

II FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S. Cl.	530/416,412,417 435/91

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

APS, USPAT, and Dialog Data Bases searched for nearness of "DNA" and the like with "union exchange" and the like and proteins.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	US, A, 4,330,464 (LAWFORD), 18 MAY 1982, See the abstract and column 2.	10,12-18
X	US, A, 4,452,734 (LARSON), 5 JUNE 1984, See column 5. and column 8.	1-18
X	US, A, 4,591,564 (WATSON), 27 MAY 1986, See column 7.	1-18
X	US, A, 4,757,134 (BLAKE), 12 JULY 1988, See column 5.	1-18

* Special categories of cited documents: 10

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IV. CERTIFICATION

Date of the Actual Completion of the International Search

07 APRIL 1990

Date of Mailing of this International Search Report

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International Searching Authority

ISA/US

Signature of Authorized Officer

for KEITH C. FURMAN Nguitho Nguyen NTO NGUYEN

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